AD_____

ISOLATING A CELL MAXIMALLY SECRETING ACETYLCHOLINESTERASE

ANNUAL REPORT

SAM ROSE
PETER C. BROWN
MAUREEN COSTELLO

July 7, 1986

Supported by
U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-84-C-4086

BIO-RESPONSE, INC.
1978 W. Winton Avenue
Hayward, California 94545

Approved for public release, distribution unlimited

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents.

SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION Unclassified	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION / AVAILABILITY OF REPORT Approved for public release; distribution unlimited
2b. DECLASSIFICATION / DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION Bio-Response, Inc. 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION
6c. ADDRESS (City, State, and ZIP Code) 1978 West Winton Avenue Hayward, CA 94545	7b. ADDRESS (City, State, and ZIP Code)
8a. NAME OF FUNDING / SPONSORING ORGANIZATION U.S. Army Medical Research & Development Command 8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER Contract No. DAMD17-84-C-4086
8c. ADDRESS (City, State, and ZIP Code) Fort Detrick Frederick, Maryland 21701-5012	10. SOURCE OF FUNDING NUMBERS PROGRAM ELEMENT NO. 61102A; PROJECT NO. 3M1-61102BS10; TASK NO. AA; WORK UNIT ACCESSION NO. 052

11. TITLE (Include Security Classification)

Isolating A Cell Maximally Secreting Acetylcholinesterase

12. PERSONAL AUTHOR(S)

Sam Rose, M.D., Peter C. Brown, Ph.D., Maureen Costello, Ph.D.

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Annual Report	FROM 1/85 TO 1/86	1986 July 7	36

16. SUPPLEMENTARY NOTATION

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	RA V, Acetylcholinesterase, Cell Culture, Cell Sorting
06	01		
06	02		

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

Techniques are described for the potential selection of overproducers of secreted protein such as acetylcholinesterase. In addition, large scale cell culture of a rodent hybridoma cell line has resulted in the production of milligram quantities of purified enzyme.

20. DISTRIBUTION / AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☐ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT. ☐ DTIC USERS	Unclassified

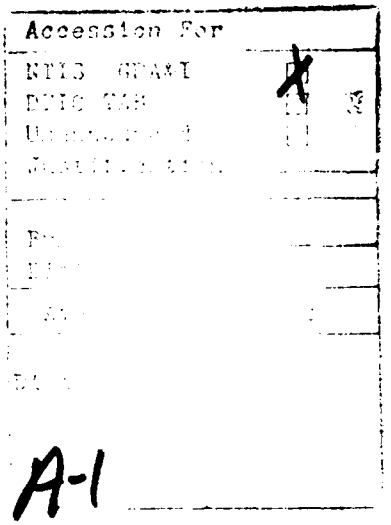
22a. NAME OF RESPONSIBLE INDIVIDUAL	22b. TELEPHONE (Include Area Code)	22c. OFFICE SYMBOL
Mrs. Virginia M. Miller	301/663-7325	SGRD-RMI-S

DD Form 1473, JUN 86 *Previous editions are obsolete.* SECURITY CLASSIFICATION OF THIS PAGE

SUMMARY

Progress in developing cell sorting techniques which are capable of selecting cells which secrete certain proteins of interest, such as acetylcholinesterase, has been made. Model systems using hybridoma cells have indicated that the fluorescence-activated cell sorter (FACS) may be used to accomplish this goal. Specifically, antibody-secreting cells were enriched using this technique from a larger population of non-secreting cells. However, this technique could not be developed sufficiently during the contract period to be brought to bear on the isolation of cells secreting acetylcholinesterase.

Project emphasis was shifted after the first year when it was realized in USAMRDC that the original goals of the project would not be met. As an alternative, we would continue development of the cell sorting approach and simultaneously provide up to 100 mg of acetylcholinesterase from a cell line that USAMRDC would provide. After approximately 60 days of growth in our large scale cell culture system, we were able to process 15 mg of product from approximately 2600 liter of conditioned medium. The final product provided to USAMRDC contained approximately 5 mg acetylcholinesterase after purification.



FOREWORD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

TABLE OF CONTENTS

List of Appendices

I. Introduction. Acetylcholinesterase is an enzyme which terminates synaptic transmission by rapidly hydrolyzing acetylcholinesterase (AChE), one of the principal neurotrans- mitters. The tonic effect of certain organic chemical warfare agents is brought about by inhibition of this enzyme.

The overall objective of this project was to conduct a research program so as to develop a source of acetylcholinesterase. Milestones in this process were to include:

(a) The development of assays for the detection of AChE;

(b) The development of a methodology for selection of cell secreting AChE (CIT);

(c) Selection of cell lines secreting AChE; and

(d) Development of production systems for AChE.

II. Quantification of AChE. Acetylcholinesterase assays utilizing a radioactive substrate are described in Appendix 1.

III. Cell Isolation Technique (CIT). While the efforts in the CIT area during the preceding year focused on two techniques for isolating cells which maximally secreted acetylcholinesterase, we have, for reasons of efficiency, focused only on the approach which utilized the fluorescence-activated cell sorter (FACS). The decision to abandon the previously described hemolytic approach was based on studies which indicated that pure ligand would be difficult to prepare and adequately test within the time frame of the contract and on dubious quantitative discrimination of the technique in general.

A. Cell encapsulation. Central to the success of the CIT approach is the encapsulation of single cells within colloidal beads less than 50 u in diameter. The small size is dictated by the fluid mechanics of the sorting apparatus of the FACS. The procedure used for cell encapsulation is described below:

PROCEDURE FOR CELL ENCAPSULATION IN AGAROSE BEADS

INGREDIENTS:

1. DOW Silicon Oil, Dimethylpolysiloxane (1000 cs)
2. 4% Agarose (Seaplaque low geling)
3. Pluronic F-68
4. DOW Silicon Oil, Dimethylpolysiloxane (5 cs)
5. Latex Beads (Interfacial Dynamics Corporation)
6. Coupled Antibody

EQUIPMENT:

1. Graduate cylinder or other suitable volume measuring device
2. Polypropylene tubes (4 ml) Falcon tubes (15 and 50 ml)
3. Balance - 30 g capacity
4. Vibromixer
5. Waterbaths (45 degree and 0 degree)

SUPPLIES:

1. pH calibration solutions, 4 and 7
2. Pipettes, graduates, beakers and Tissue Culture Water (TCW) filled wash bottles as needed

PROCEDURE:

A. Encapsulation of Cells

Place 25ml of 500cs oil (DOW silicon, dimethylpolysiloxane 1:1 mixture of 1000 cs and 5 cs) in a 50 ml Falcon tube and place in 45°C water bath. Place a tube of 4% Agarose in

saline (FMC, Seaplaque low geling) in 70°C water until it melts, then place it also in the 45°C water bath. Place 1-3 x 10^7 cells suspended in 900 ul of PBS in a 4 ml polypropylene tube and add 20 ul of F-68 pluronic (BASF, 10 mg/ml in saline), invert the tube to mix, and place it in the 44° ±2°C water bath. Next add 1 ml of the agarose to the PBS/Pluronic and vortex mix while keeping tube warm. Pour the warm oil into a 50 ml beaker set in the vibromixer jig. Turn the vibromixer power to 110 volts and add the agarose suspension drop-wise to the oil (taking no longer than 5 sec.) and allow the two phases to emulsify for 120 ± 5 sec. Now add 25 ml of -20° ±5°C 5 cs oil (DOW silicon) while still mixing and place an ice bath around the emulsion beaker. Allow the emulsion to setup for 5-10 min. Pour the emulsion back into the 50 ml Falcon tube and centrifuge (300g, 3 min.), pour off the oil phase and add 10 ml of PBS or MEM and vortex mix until the agarose beads are resuspended. Pour the agarose beads into a 15 ml falcon tube and centrifuge, decant off the overlaying oil and media. Using a new 15 ml tube resuspend the agarose beads in 10 ml of PBS or MEM and centrifuge. Pour off the overlaying media and finally resuspend beads in media.

B. Encapsulation of Cells and Latex

Place 25 ml of 500 cs oil (DOW silicon, dimethylpolysiloxane) in a 50 ml Falcon tube and place in 45°C water bath. Place a tube containing 1 ml of 3% Agarose (FMC, Seaplaque low geling) in 0.3M Sucrose (isotonic) pH 7.0 buffered with 4mM phosphate in 70°C water until it melts, then place it also in the 45°C water bath, add 100 ul of Pluronic F-68 solution (1% w/v in deionized water).

Prepare a cell suspension as in procedure A and mix with 400 ul latex suspension and warm to 37°C. Next pipette the cell/latex suspension into the agarose and vortex mix. The emulsion oil is now poured into the vibromixer jig and agitation is started. The agarose/cell suspension is next poured into the emulsion oil and vibromixer power brought up to 110 volts. Emulsify for 2 minutes, then add 20 ml of -20°C, 100cs oil and continue to mix for 5 seconds. Stop agitation and allow emulsion to set in ice bath for 20 minutes. Pour the emulsion over 10 ml of MEM in 50 ml centrifuge tube and centrifuge for 5 minutes at 350g. Decant the oil phase and wash the resulting beads once more in MEM.

C. Encapsulation of Cells and Coupled Antibody

As in procedure A except that 1 mg of dextran antibody conjugate in 200 ul PBS is added to the 900 ul PBS cell suspension and this mixture is encapsulated.

While considerable efforts were expended on this aspect during the first year of the contract, reproducibility and recovery of cells continued to plague the process. As a result of more than 30 experiments during year two of the project, the process has been brought under control. It is now possible to achieve over 80% encapsulation and recovery of cells in beads under 100 u in diameter (Figure 1A).

B. Insolubilization of trapping antibody. In order to prevent secreted cell products from leeching out of the agarose beads, a trapping antibody, specific for the secreted product such as acetylcholinesterase, must be immobilized within the beads containing cells.

Initial experiments attempted to utilize colloidal gold particles 20-30 nM in diameter to which antibody or other protein spontaneously adheres. Because of the irreproducibility of the technique and the difficulty of physically handling these particles, this approach was abandoned in favor of carboxylated latex spheres, 1 u in diameter, to which antibody was covalently coupled using the technique of Molday et al (1). After coupling, the derivatized latex spheres were characterized for antigen binding capacity at saturation.

In practice, cells along with these derivatized particles are coencapsulated within the bead. This results in a bead with, on average, 1 cell and about 50 particles. These particles do not appreciably contribute to the light scattering or alter the physical characteristics of the bead itself. Representative beads with cells and fluorescein-labeled latex particles are shown in Figure 1B.

C. Model studies. In order to establish the basic parameters associated with this approach to CIT, two types of cells were initially employed in model studies. The first cell type chosen was a mouse hybridoma cell line which secreted a monoclonal IgG_1 to an unknown antigen. The secretion rate was approximately 1000 immunoglubular molecules/cell/second. Because of its high rate of secretion, this cell would, in theory, give the greatest signal to noise ratio during the actual sort. The second cell type investigated was a human melanoma cell line which secretes tissue plasminogen activator at a rate of approximately 10 molecules/cell/second. Thus, with a 100-fold lower secretion rate, this cell represents a significantly greater challenge and it approaches levels seen for acetylcholinesterase (AChE) secretion. Both cell lines were proprietary to Bio-Response.

1. Murine hybridoma. The goal of this model study was to determine whether the system was capable of identifying and physically isolating a hybridoma cell which secretes a monoclonal antibody from a significantly greater population of cells which do not secrete that product. During this phase we only wished to determine whether the physical aspects of sorting could be established; no actual expansion of the sorted population was to be attempted.

   The mouse hybridoma cells were mixed with a human Jurkat cell at a ratio of 1:20, thus giving a population of which only 5% of the cells secreted a monoclonal antibody. In addition, the hybridoma population was prelabeled overnight with [^{14}C]-thymidine to enable identification of these cells by autoradiography both before and after the separation. To capture the secreted monoclonal antibody, carboxylated latex spheres (Polyscience, Ind., Warrington, PA) were derivatized with goat anti-mouse IgG (Cooper Biomedical, Malvern, PA).

   Following coencapsulation of the reconstructed population with derivatized particles, cells (i.e., beads) were incubated overnight in

medium to allow for accumulation of antibody and absorption to the immobilized antibody. Next, cells (beads) were washed and incubated with fluorescein-labeled goat anti-mouse IgG and after 1 hour excess antibody washed free of the beads. The optimal amount of this second antibody used was determined in reconstruction studies (not shown). Finally, the population was interrogated by FACS. From the distribution of fluorescent beads shown in Figure 2, the uppermost fluorescent 5% of the population was sorted from the rest of the population. Cells from these selected beads were isolated, fixed on microscope slides, and processed for autoradiography using Kodak NTB-3 emulsion. After a suitable exposure period, the slides were developed and stained to reveal nuclei, and labeled nuclei were counted and compared to unlabeled nuclei in both the selected population and the starting, unsorted population. These data are displayed in Table 1. Whereas the presorted (starting) population was composed of, as planned, 5% antibody-secreting cells, this percentage was increased to 54% in the post-sort population. In other words, the sort resulted in a 10-fold enrichment of antibody-producing (i.e., ^{14}C-labeled) cells. We feel confident that the unlabeled cells present in this population could have been reduced still further if a greater degree of discrimination (i.e., 1% vs 5%) was employed. Nevertheless, this represents the first concrete demonstration that the approach actually works. These results were obtained in January 1986.

2. Human melanoma. Initially, a protocol virtually identical to that used in Model 1 was used and showed that the system could also enrich for cells which secrete the t-PA. Naturally, different antibody reagents were employed. Specifically, goat antiserum to the protein of interest was both immobilized to the latex particles and fluoresceinated for use as a second, labeled antibody.

   A second experiment was performed most recently in which the uppermost fluorescent 10% of

encapsulated melanoma population was sorted, liberated from the beads, expanded for analysis, and resorted three times. These results showed a 1.8-fold increase in relevant protein secretion per cell after multiple sorts.

D. High producers of AChE. Because the CIT technology took significantly longer to develop than originally proposed, we were unable to adequately characterize the approach before the expiration date of the contract.

IV. Production of AChE. The original contract called for the development of a cell line which made upwards of 1% of its total protein as AChE and the production from that line of 100 mg of purified AChE. Since it was apparent that unexpected technical difficulties in the development of CIT virtually precluded development of a high producing cell line within the contract period, it was determined that Bio-Response would receive a newly developed cell line from Dr. Ken Hunter (Uniformed Services University of the Health Sciences, Department of Pediatrics) which secretes relatively large quantities of AChE and Bio-Response would attempt to produce up to 100 mg of AChE from that line. In addition, work would continue toward the development of a CIT.

A. Arrival of cell line and initial characterization. The cell lines designated E-2SA3 and E-2 clone D-6 were received May 22, 1985. Each were derived from a hybridoma formed by the fusion of rat hepatoma cell line (FRL-8A6R) and a mouse hepatocyte (Balb/c). Whereas the D-6 clone was isolated by simple dilution, the SA3 line was derived by soft agar cloning.

After an initial quarantine and mycoplasma screen, the cell lines were compared for their ability to grow in defined, serum-free medium and to secrete AChE as measured by radiometric assay using [^{14}C] acetylcholine as substrate (2) (Appendix 1). The necessity of growing cells in the absence of fetal serum was indicated by experiments which showed that high levels of AChE were found in all lots of fetal calf serum tested (data not shown).

For evaluation of cells and medium, cells were initially plated, in basal medium (1:1 mix of Dulbecco's Modified Eagles' Medium and F-12 (Coon's) containing 5% heat-inactivated newborn calf serum (NCS) (Gibco)). The choice of heat-inactivated NCS was also based partially on the data displayed in Figure 3., and also because of the reduced expense and greater availability of NCS as compared to fetal calf serum. Figure 3 shows that the endogenous AChE activity of NCS was dramatically decreased with heat treatment at 56^{o}C for 30 minutes and eliminated at 60 minutes. In addition, the AChE activity in the untreated sample was some 3-fold lower than those levels seen with fetal calf serum (data not shown).

The ability to inactivate endogenous AChE levels in calf serum is important because this inactivation obviates the distinct possibility that observed levels of secretion of AChE were merely exocytosed bovine serum AChE.

It has been the goal of all production contracts at Bio-Response to grow all cell lines in the minimal amount of protein supplement. To this end, we have developed a proprietary serum-free, defined medium which, at normal dilution, contains only 40 ug of total protein/ml of medium. This medium designated DLV was evaluated along with other media containing no protein supplement whatsoever. These data are displayed in Table 2 along with a comparison of the two cell lines from Hunter's group and supernatant from the mouse neuroblastoma cell line NB41A3, a derivative of the C1300 mouse neuroblastoma which is known to secrete AChE (see American Type Culture Collection catalog, 95). These data show that of all the combinations tested, the E2 clone D grown in DLV was most productive with respect to AChE secretion. Furthermore, the level of secretion from the D-6 clone is approximately 3 x $10^{-10}/10^{6}$ cells/24-hour period or about 5 x 10^{-9}/average roller bottle/day. This level of secretion was a full 10-fold higher than any of the human AChE-producing cell lines tested. Finally, inhibitor studies revealed that the esterase activity was truly specific for acetylcholine (see Figure 4).

B. Pilot study for large scale production. Because the cell line, clone D, grows in the anchorage-dependent mode, we chose a substrate consisting of packed glass beads (3 mm) contained within a perfusion vessel of 4 liters capacity and perfused continuously with oxygen-rich, nutrient-supplemented medium. This system and a similar design have been described and will not be elaborated upon herein (3,4).

Because it was noted in earlier static studies that the clone D cells required periodic supplementation with heat-inactivated serum in addition to continuous DLV, a pulsed mode of serum delivery to the perfused bioreactor was decided upon. In this mode, cells were seeded in 5% serum, switched to DLV for 7 days, pulsed for 8 hours only with 5% serum, and returned

to serum-free growth. This cycle was repeated every week for about 5 weeks. Product was saved only during the period of serum-free growth, allowing for a 12-hour washout period after the pulse of serum. The results from the pilot study are shown in Figure 5.

Towards the end of the study, during which period the cell densities were greatest, the effects of the pulsed serum protocol were most evident. After the pulse, production increased significantly, followed by a decline phase. Over the course of the pilot study, approximately 6.6 x 10^7 pg/IU AChE were collected which, at a conversion of 20 pg/mIU, equals approximately 1.2 mg.

C. Large scale production. With the results from the pilot study in hand, large scale production was undertaken with the assemblage and inoculation of five-12-liter glass bead units on October 18, 1985. The bioreactors were operated on the pulsed serum mode until January 7, 1986. Production data from these bioreactors are shown in Figure 6. Data are presented in micrograms per day per unit using the conversion factor mentioned previously. Cumulative production for all units over the 9-week production period was approximately 15 mg collected in 2600 liters of cell-free supernatant. If this amount of product were to have been produced in conventional roller bottles, we estimate that 60,000 separate harvest would have been required to produce 25 mg of product. This estimate is based on extrapolation from the production of AChE from conventional T-flasks (60 IU/25 cm^2 flask/24-hour period). The level of production achieved in our continuous perfusion system surprised our scientists who projected total production based on static culture studies. We suspect, but have not shown, that continuous perfusion significantly enhanced AChE secretion on a per cell basis and resulted in the collection of product some 10 to 20-fold greater than that originally though possible from the five 12-liter units.

D. Product concentration and purification. For volume reduction, 400-liter lots of pooled bioreactor supernatant were concentrated approximately 120-fold

using a Millipore Pelicon cassette (30,000 molecular weight cutoff). The concentrate (approximately 5 x 10^4 IU/ml) was then adsorbed in batch with the procainamide affinity gel (supplied by Affinity Biosystems, La Jolla, CA) at a ratio of approximately 2 liters of concentrated supernatant to 200 ml of affinity matrix. Elution of adsorbed material was monitored by UV adsorbtion spectrophotometry. Pooled material collected in the eluted peak was desalted over G-25 Sephadex (Pharmacia) equilibrated in phosphate buffer (40 mM Na_2HPO_4, pH 8.0, 50 mM NaCl). Further concentration of the material from the desalting column was by ultrafiltration over hollow fibers and modules normally used for kidney dialysis (10,000 MWCO) preadsorbed with human serum albumin (1%). This final concentrate was sterile filtered and made 50% in sterile glycerol. The final product was supplied at a concentration of 314 units/ml in a volume of 750 ml. Using a conversion of 20 ng/IU, the final product is at 6.3 ug/ml or 4.74 mg in total.

We estimate that the unconcentrated cell-free supernatant contained approximately 15 mg and that the losses upon concentration and purification were in the order of 75%.

E. Characterization of final product. The specificity of the final product was established through the use of specific (BW284C51) and nonspecific tetraisopropyl pyrophosphoramide (ISO-OMPA) inhibitors of AChE both in the radiometric assay and in fluorescence zymography of samples electrophoresed through 6% native polyacrylamide gels (Dr. R. Schuman, personal communication). In Figure 4A, the specific identity of final product as AChE is established by this approximate 4 log shift in I_{50} values when enzyme assays were performed in the presence of varying concentrations of either inhibitor. Of interest is the enhanced sensitivity of rodent AChE to the specific inhibitor when compared to the enzyme from chicken (I_{50} - 1 x 10^{-8}M vs 5 x 10^{-6}M) (5). For comparison, in Figure 4B similar inhibitor studies were performed with human AChE obtained from red blood cells (Sigma).

As an independent confirmation of specificity and in an attempt to gain insights into the structure of the enzyme, various samples were electrophoresed through 6% native polyacrylamide gels and stained for esterase activity using the fluorometric substrate N-methyl indoxyl ester (Dr. Schuman, personal communication) (6). Figure 7 shows three gels loaded and run identically and in parallel. Lane 5 with purified final product shows a major and minor esterase band, both of which are extinguished by the specific inhibitor (gel B) but not the nonspecific inhibitor at a 100-fold greater concentration. This behavior is shared with the human AChE (lane 4) but is different from horse serum butyrylcholinesterase (lane 3). Not visible in the gel reproductions are faintly staining bands in NCS lanes (lanes 2) which appear to be specific for AChE and completely disappear upon heat treatment (lane 1). This latter result confirms data presented in Figure 3. Finally, none of these data indicate probable form or molecular weight of the final product although Rick Schuman, a co-developer of the cell line along with Dr. Ken Hunter, believed the major form to be a G_4 structure of about 330,000 daltons.

A final experiment to detect possible contamination of concentrated product with bovine AChE was performed. A monoclonal antibody, AE-1, reacts with bovine and human forms of AChE, but not the rat or mouse species of protein (Figure 8). The final product was treated according to the protocol in Appendix 3.

Bovine serum AChE was detectable, but no activity could be obtained from the final product. The major and minor bands obtained after gel electrophoresis and reaction with N-methyl indoxyl acetate are not due to the presence of active bovine AChE.

F. Disposition of final product. The purified material was sent to Col. Jerry Sadoff, Department of Bacterial Disease, Walter Reed Army Institute of Research, Building 40 - Room 2085, Washington, D.C. 20307.

REFERENCES

1. Molday, R.S., Dreyer, W.J., Rembaum, A., and Yen, S.P.S. (1975). J. Cell Biol., 64, 75-88.

2. Rotundo, R.L. and Fambrough, D.M. (1980). Synthesis, transport and fate of acetylcholinesterase in cultured chick embryo muscle cells. Cell, 22, 583-594.

3. Brown, P.C., Costello, M.A.C., Oakley, R., and Lewis, J. (1985). In: Large-Scale Mammalian Cell Culture, editors W. Tolber and J. Feder, Academic Press, pp 59-71.

4. Whileside, J.P., Whiting, B. R., and Spier, R.E. (1979). Development of a methodology for the production of foot-and-mouth disease virus from BHK21C13 monolayer cells grown in a 100 L (20 M^2) glass sphere propagator. Dev. Biol. Standard., 42, 113-119.

5. Rotundo, R.L. and Fambrough, D.M. (1979). Molecular forms of chicken embryo acetylcholinesterase in vitro and in vivo: isolation and characterization. J. Biol. Chem. 254, 4790-4799.

6. Guilbault, G.G. and Sadar, M.H. (1968). N-Methyl indoxyl esters as substrates for cholinesterase. Anal. Lett. 1, 365-379.

Table 1. Enrichment of antibody-secreting cells after sorting

Sample	Total Cells	Labeled Cells	Percent Labeled
8501 ^{14}C cells	200	200	100
Pre-sort population	300	20	5
Post-sort population	294	160	54

8501 Mouse hybridoma cells prelabeled with [^{14}C] thymidine were mixed (1:20) with a nonsecreting human Jurkat cell line. Percent refers to the fraction of sorted cells which have incorporated label, i.e., are the desired 8501 cells, as determined by autoradiography.

Table 2. Comparison of cell lines in various media formulations

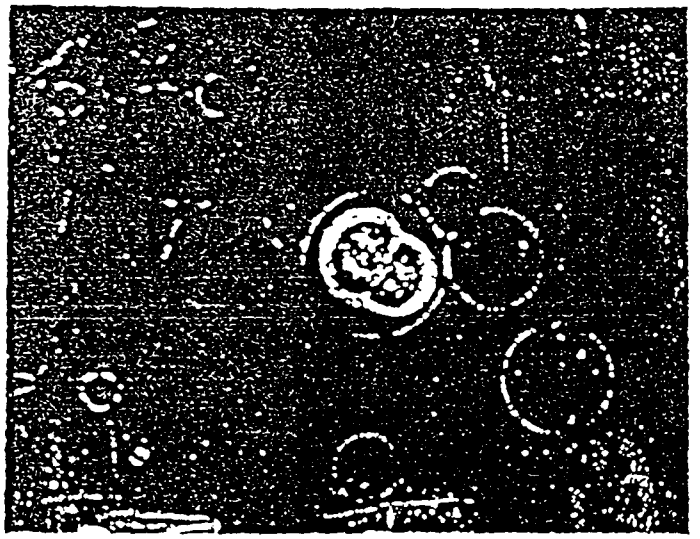
Cell line	AChE activity[a] (mIU/10^6 cells/24 hr.) Protein-free	DLV
E2SA3	3.7 3.2	5.3 4.7
E2clone D	7.6 5.5	14.3 13.8
NB41A3 (ATCC-CC! 147)	N.A.[b]	0.9

Cells were plated in 5% heat-inactivated NCS in basal medium. After 48 hours, medium was changed to either protein-free or DLV. After 72 hours, medium was changed and subsequent 24-hour collections harvested for assay (one collection only for NB41A3).

(a) AChE activity by radiometric assay using a standard of human erythrocyte AChE (Sigma No. C5400) solubilized in buffer with 50% glycerol and stored at -20°F.

(b) Not applicable.

A.



B.

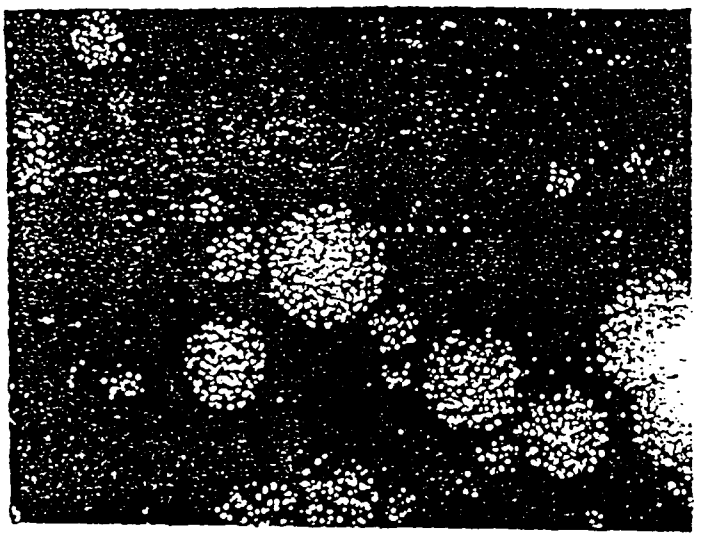


Figure 1. Encapsulation of cells without (1A) and with fluoresceinated latex particles (1B). Cells were encapsulated as indicated in the text.

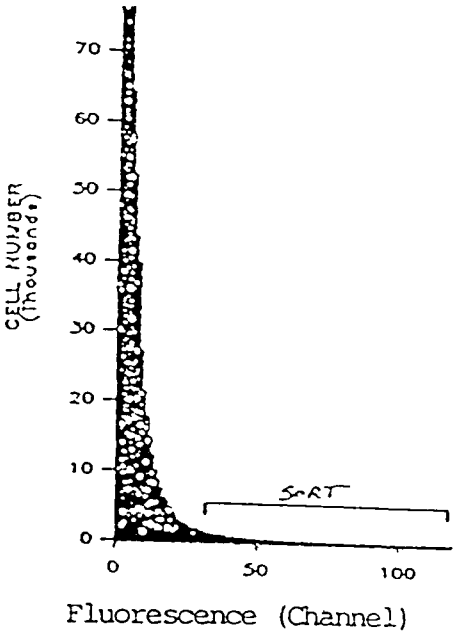


Figure 2. Model System 1: fluorescence distribution and sort criteria. A 1:20 mixture of 8501 murine hybridoma cells and human Jurkat cells was encapsulated and sorted using the FACS instrument. Channels 27-124 which comprised the uppermost fluorescent 5% of the population were sorted and processed for autoradiography (see Table 1).

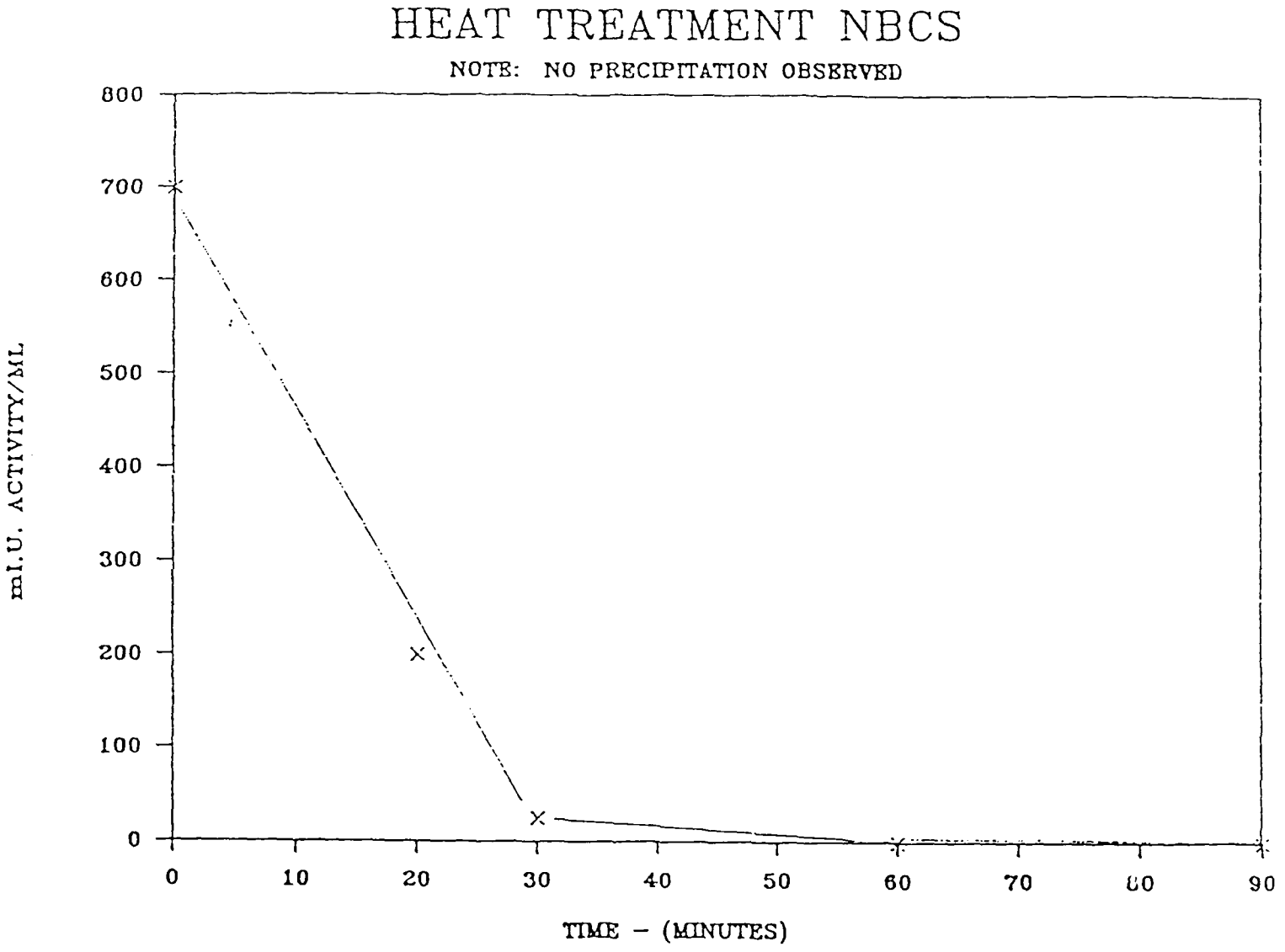


Figure 3. Heat treatment of NBCS. Aliquots of NBCS were incubated for varying lengths of time at 56°C. AChE assays (radiometric) were performed on each aliquot at the end of 90 minutes.

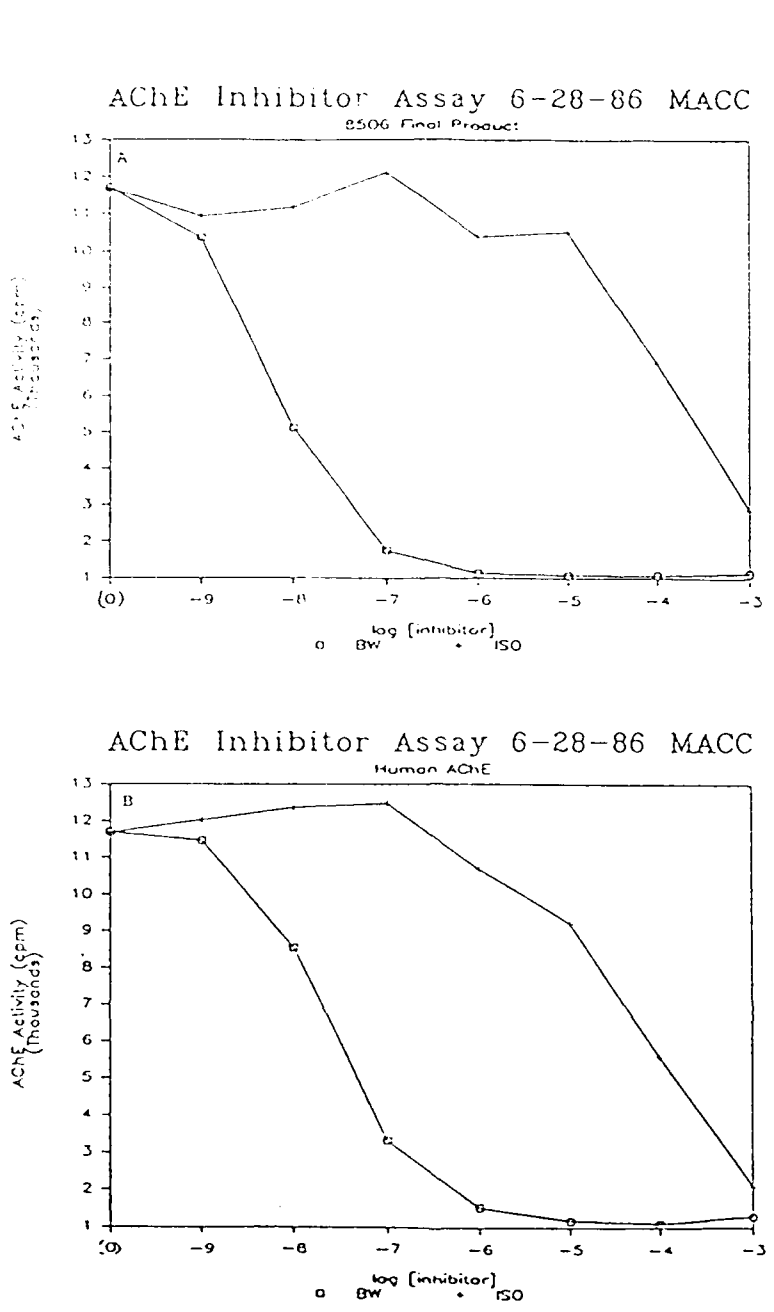


Figure 4. Effects of specific and nonspecific AChE inhibitors. A, samples of purified final product were assayed for AChE activity in the radiometric assay in the presence of varying concentrations of specific inhibitor (BW284C51) and nonspecific inhibitor (ISO-OMPA). The I_{50} values for the specific inhibitor were approximately 1 X 10^{-8}M; for the nonspecific inhibitor the corresponding value was approximately 1 X 10^{-4}M. B, radiometric assays were performed with commercially available human AChE (Sigma).

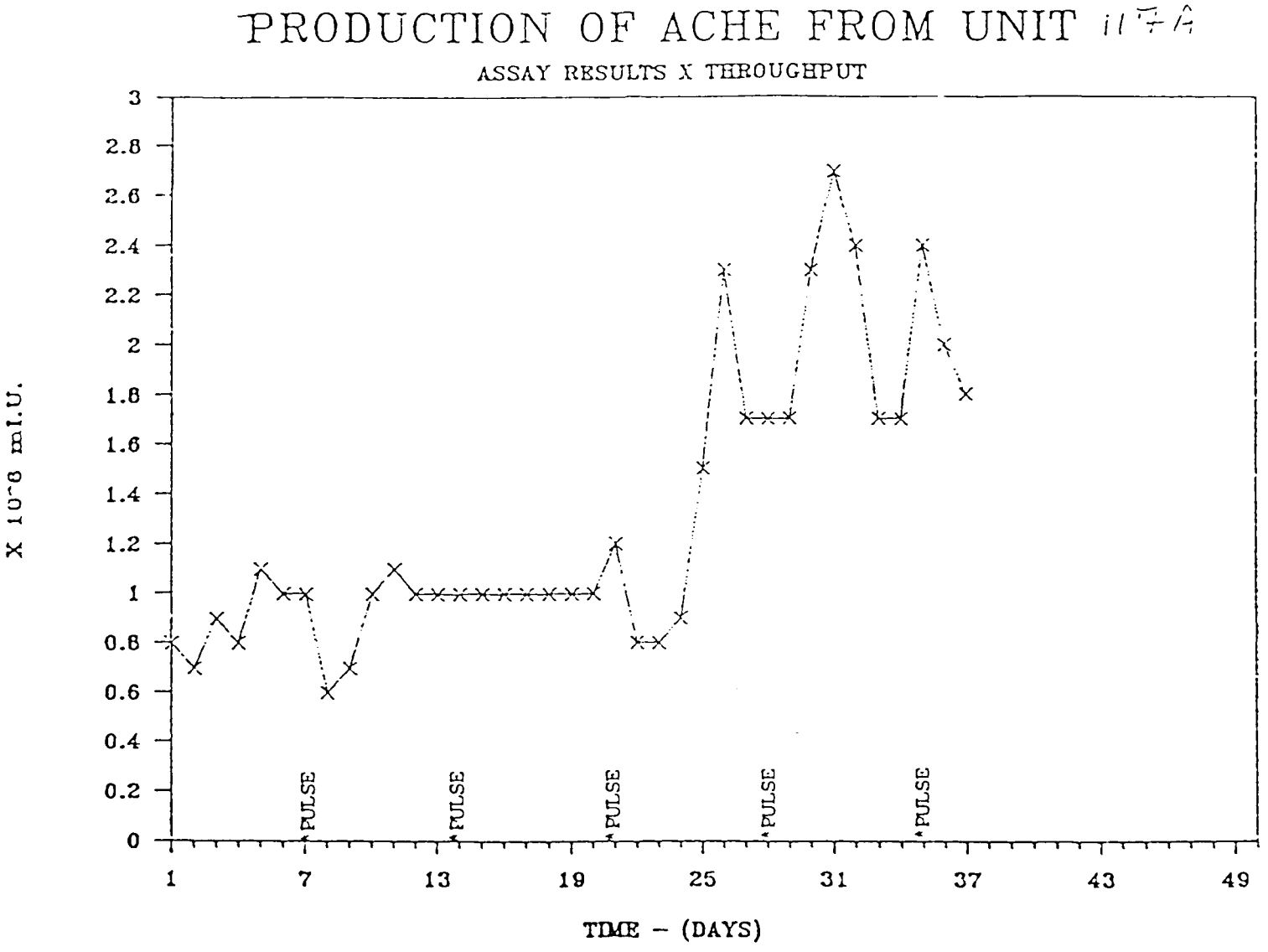


Figure 5. Production of AChE from unit 117A. A 4-liter packed glass bead unit (117A) was inoculated with cells on day 1. At weekly intervals, the unit was pulsed with medium containing 5% heat-inactivated newborn calf serum. AChE assays were performed and the data normalized to total daily AChE in IU.

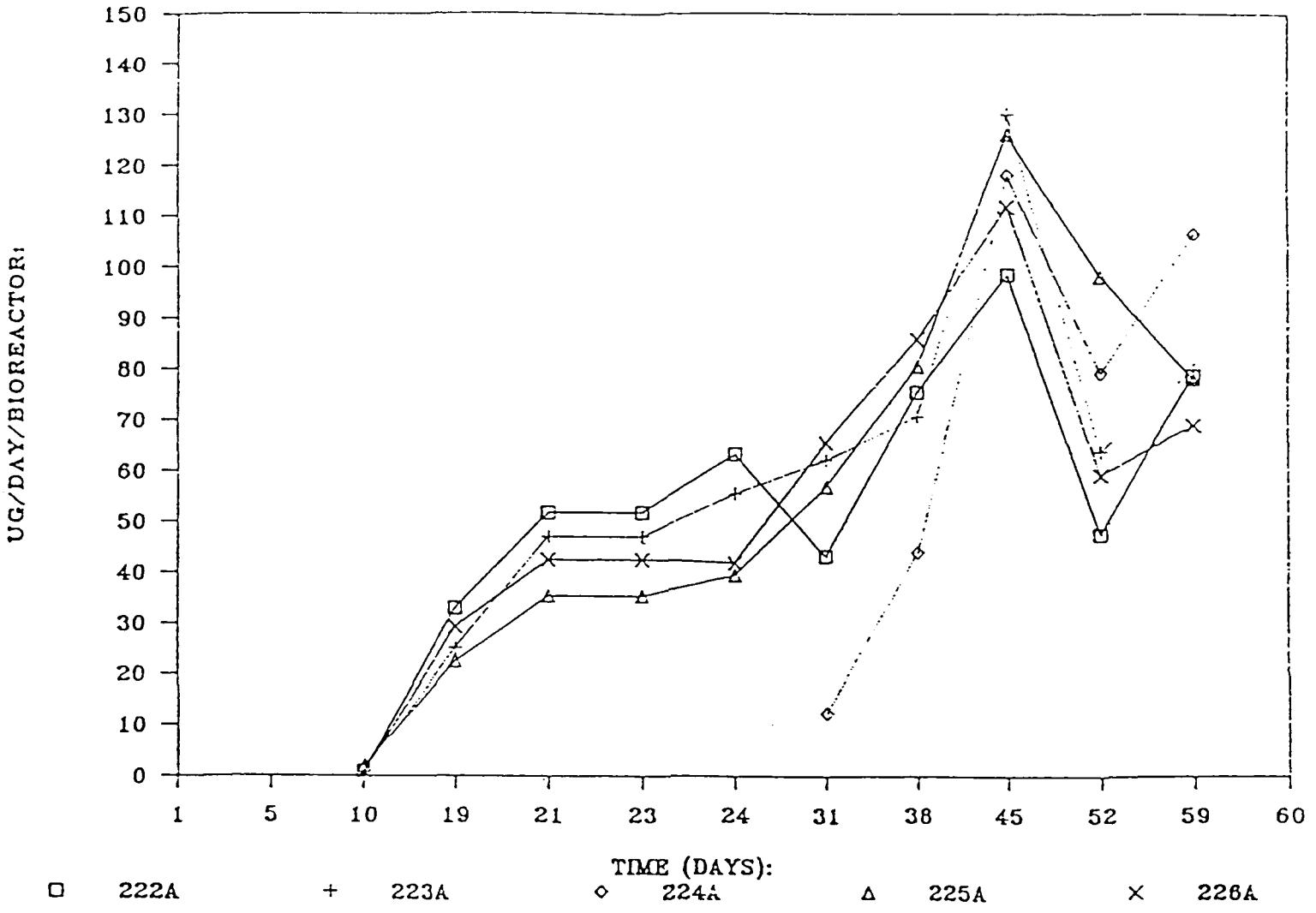


Figure 6. Production of AChE. Five 12-liter packed glass bead units (No. 222A-226A) were operated as indicated in Figure 5. Cell-free supernatants were prepared and purified as indicated in the text.

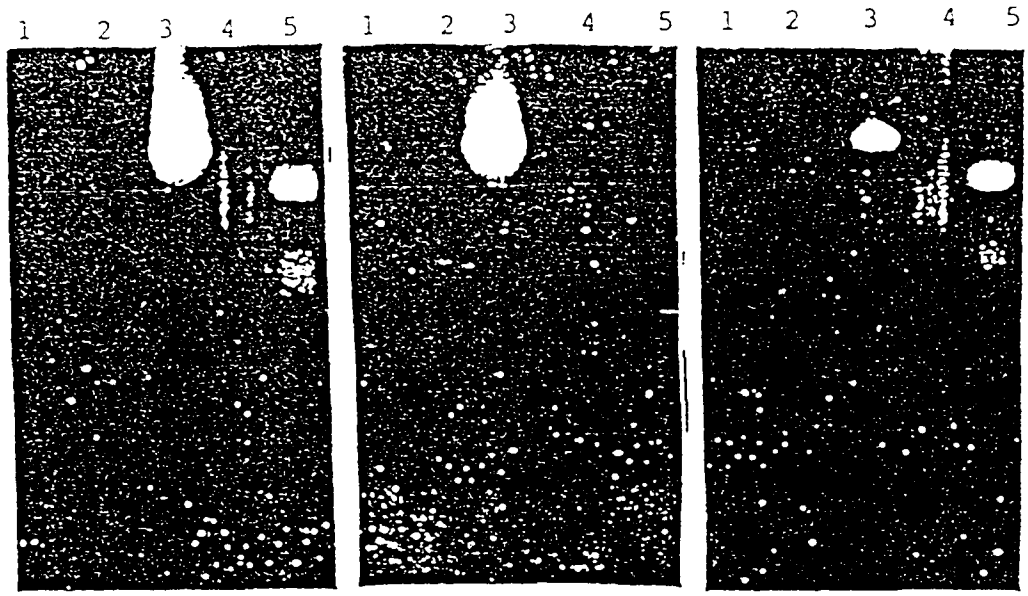


Figure 7. Identification of AChE activity by electrophoresis and fluorescence zymography. Various samples were electrophoresed through 6% polyacrylamide gels in the absence of sodium dodecyl sulfate (SDS) and reducing agent in TRIS-glycine buffer, pH 8.3. Subsequently, the gels were stained with N-methyl indoxyl acetate (2×10^{-3}M) for 30 minutes at 37°C in the dark, transilluminated with a long-wave ultraviolet light source, and photographed. Lane 1, heat-treated newborn calf serum, 15 ul; lane 2, newborn calf serum, 15 ul; lane 3, serum butyrylcholinesterase, 15 IU; lane 4, human red blood cell AChE, 40 IU; lane 5, purified final product, 15 IU. Gel A, native; gel B, preincubated in BW284C51 (1×10^{-7}M); gel C, preincubated in ISO-OMPA (1×10^{-5}M).

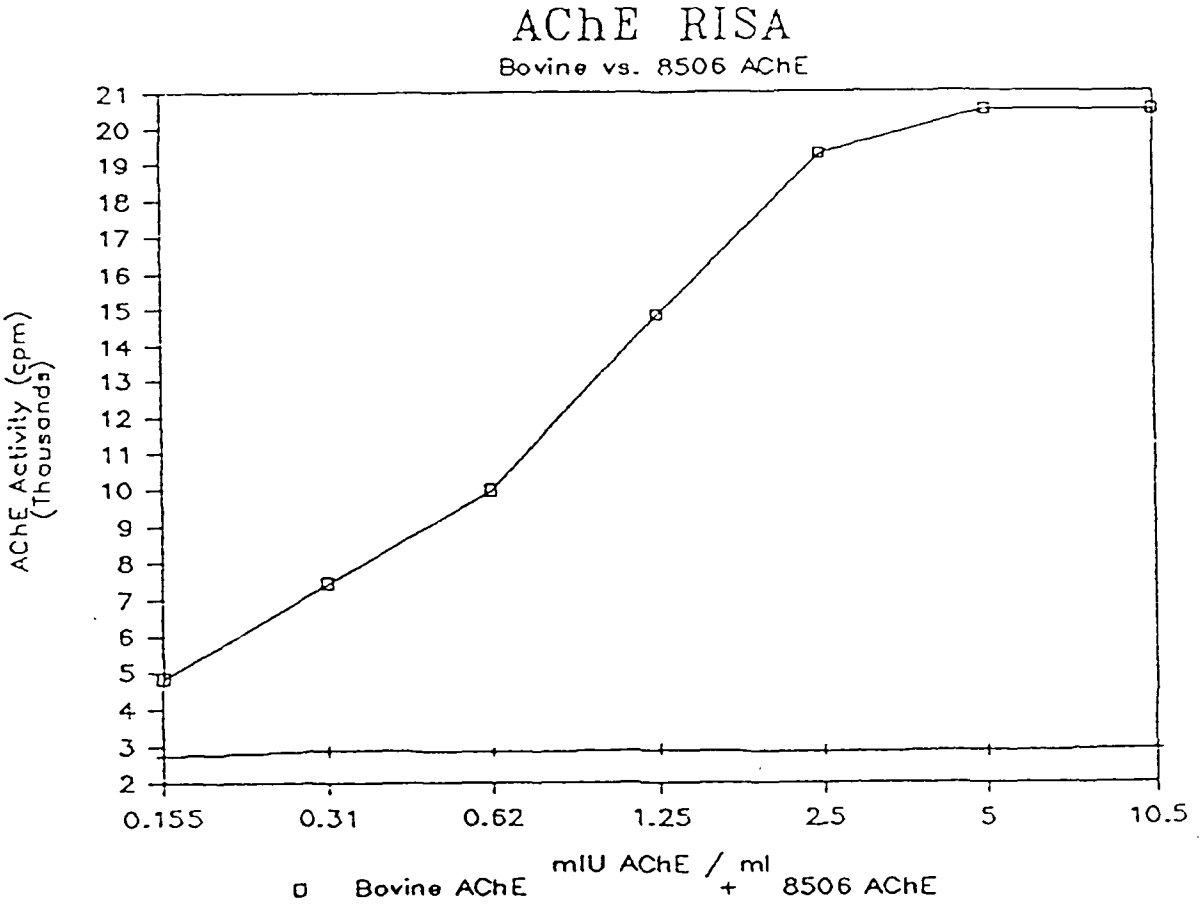


Figure 8. Reactivity of final product in radiometric immunoadsorbent solid-phase assay (RISA). AE-1-monoclonal antibody recognized both human and bovine AChE but not the rat or mouse species of AChE (data not shown). Samples for final radiometric assay were preincubated with the AE-1-antibody to isolate activities due to bovine AChE. A standard prepared with bovine serum was tested in comparison to final product.

DISTRIBUTION LIST

1 Copy	Commander US Army Medical Research and Development Command ATTN: SGRD-RMI-S Fort Detrick, Frederick, Maryland 21701-5012
5 Copies	Commander US Army Medical Research and Development Command ATTN: SGRD-PLE Fort Detrick, Frederick, Maryland 21701-5012
2 Copies	Defense Technical Information Center (DTIC) ATTN: DTIC-DDAC Cameron Station Alexandria, VA 22304-6145
1 Copy	Dean School of Medicine Uniformed Services University of the Health Sciences 4301 Jones Bridge Road Bethesda, MD 20814-4799
1 Copy	Commandant Academy of Health Sciences, US Army ATTN: AHS-CDM Fort Sam Houston, TX 78234-6100

APPENDIX 1

RADIOMETRIC AChE ASSAY PROTOCOL
6-27-86 P.S. & MACC

PURPOSE: To quantitate acetylcholinesterase (AChE) concentration using a radioactive substrate.

REACTION:

$$\text{14C-acetylcholine} \xrightarrow{+\ \text{AChE}} \text{14C-acetate} + \text{choline}$$

Since the 14C-Acetate is organic, it partitions into the organic phase in an aqueous/organic extraction. Therefore counts in the organic phase represent reacted acetylcholine thus acetylcholinesterase.

REAGENTS/MATERIALS:

1. 14C-Acetylcholine Chloride (Amersham Code CFA.310)
   Prepare aliquots as follows:

   1 vial + 2.5 ml 4mM acetylcholine chloride (see below) + 2.5 ml "40X". Aliquot in 200 ul portions and freeze dry (use radiation trap). To use, 1 aliquot is brought up to 4.0 ml in reaction buffer. (Thus it is used at 2x).

2. Reaction Buffer:

   100mM sodium phosphate, pH 7.0
   150mM sodium chloride
   0.5% Triton X-100
   0.25mM EDTA

3. Stop Buffer:

   50mM glycine (1.88g/500ml))
   1M NaCl (29.2g/500ml)
   pH 2.5 using HCl

4. Scintillation Cocktail:

   400 ml isoamyl alcohol (Mallinckrodt #2992070)
   17.2g PPO (Amersham #190020)
   1.0g POPOP (Amersham #190050)
   + toluene to make 4L (Mallinckrodt #8608070)

5. Acetylcholinesterase Standard:

   Sigma Human AChE #C-5400
   Prepare 100 ul aliquots at 1 u/ml in 1.0% BSA, 0.5% Triton X-100, 50% glycerol in PBS, pH 7.

6. Cold acetylcholine chloride (Sigma #420-750 or equivalent).

7. 10 x PBS.

8. 0.5% Triton X-100.

9. 12 x 100 mm polypropylene tubes and caps (Fischer #14-956-7E and 14-956-15).

PROCEDURE:

1. Pretreat samples:

   10 ul 0.5% TX-100 + 10 ul 10X PBS + 80 ul sample. This results in a 1.25 x dilution.

2. Make further sample dilutions in reaction buffer. Be sure to take the 1.25 x dilution into account.

3. Prepare standard dilutions:

10 ul 0.5% TX-100 + 10 ul 10 X PBS + 80 ul 1 u/ml AChE = 800 mU/ml
To the above, add 700 ul reaction buffer = 100 mU/ml
200 ul @ 100 mU/ml + 1.80 ml reaction buffer = 10 mU/ml

Test at	ul 10mu/ml	ul reaction buffer
5 mU/ml	500	500
4 mU/ml	400	600
3 mU/ml	300	700
2 mU/ml	200	800
1 mU/ml	100	900
0.5 mU/ml	50	950
0.0 mU/ml	0	1000

4. Place 50 ul sample in a 12 x 100 mm polypropylene tube.

5. Add 50 ul 2 x 14C-acetylcholine chloride (see reagents #1).

6. Cap tube and place in 37^{o}C incubator for 2 hours.

7. Stop reaction by adding 2 ml stop buffer. Immediately add 5 ml scintillation cocktail, cap and shake vigorously.

8. Centrifuge at 1000 rpm for 10 minutes in the tabletop centrifuge (equipment room).

9. Using a p5000 pipetman, carefully transfer 4.5 ml of the organic (upper) layer to a scintillation vial - label CAP of vial.

10. Allow vials to sit under the yellow cover of the scintillation counter a few hours before counting. This reduces chem-iluminescence.

11. To count,

Push	EDIT R
Response	Parameter Group
Push	04 R
Response	ID
Push	Date (Month, Day, Year)
Response	Line #

You can ignore the remaining responses by pushing "STOP R", wait for the machine to respond with PASO, then push "START 04 R". Make sure there is an empty rack (no carrier vials, etc.) behind your samples. This tells the counter to STOP.

12. Use the CURVE FITTER - PC program to process data.

APPENDIX 2

PROTOCOL 9-30-85 P.S.

PURPOSE: To quantitate AE-1 antibody-specific acetylcholinesterase in cell-free supernatants.

THEORY: AE-1 antibody is anti-human-acetylcholinesterase. Therefore, it will bind to human AChE and an cross- reacting AChE (example bovine). AChE cleaves acetyl choline into acetate and choline:

$$\text{14C-acetylcholine} \xrightarrow{+\text{acetylcholinesterase}} \text{14C-acetate} + \text{choline}$$

Since the 14C-acetate is organic, it partitions into the organic phase in an aqueous/organic extraction. Therefore counts in the organic phase represent reacted acetylcholine thus acetylcholinesterase.

REAGENTS/MATERIALS:

1. AE-1 antibody (prepared at Bio-Response, purified at Bio-Rad) "peak 5" = 4.7 mg/ml.

2. Acetylcholinesterase (AChE) standard: Human AChE = Sigma #C-5400. Dilute to 1 unit/ml in 1.0% BSA 1.0% Triton X-100 0.02% sodium azide in PBS, pH 7.3, + 50.0% glycerol (final concentration BSA = 0.5% Triton X-100 = 0.5% azide = 0.01%). Store at -20°C.

3. Cold acetylcholine chloride (Sigma #420-750 or equivalent).

4. 14C-Acetylcholine chloride (Amersham Code CFA.310). 1 vial + 2.5 ml 4 mM acetylcholine chloride = 2.5 ml "40 x". Aliquot in 200 ul portions and freeze dry (use radiation trap). To use, 1 aliquot is brought up to 8.0 ml in buffer A = 8.0 ml @ 1 x.

BUFFERS/SOLUTIONS:

1. Hepes Buffered Saline (HBS) pH 7.4 + 0.02% sodium azide:

20 mM HEPES Sigma #H-3375	MW=238.31	4.77 g/L
150 mM sodium chloride	MW= 58.4	8.76 g/L
0.02% sodium azide 1 ml 1000x/L, 100x=20g/100ml		

2. "Buffer A" = PBS + 0.25 mM EDTA + 0.05% Triton X-100 + 0.02% sodium azide, pH 7.3

3. PBS + 0.05% Triton X-100 (Sigma) pH 7.3 (3.0 ml Triton/6L PBS)

4. 1.0% BSA Fraction V (U.S. Biochemical #1085/or equivalent 5g/500ml)
0.2% gelatin (1g/500ml)
0.02% sodium azide (0.5ml 1000x/500ml)
pH 7.3
"BSA/GEL/PBS"

5. "Stop" Buffer
50 mM glycine MW = 75.07 1.88g/500ml
1 M sodium chloride MW = 58.44 29.2g/500ml
pH 2.5 using 1-5 M HCl

6. Scintillation Cocktail
400 ml isoamyl alcohol (Mallinckrodt #2992070)
17.2g PPO (Amersham #190020)
1.0g POPOP (Amersham #190050)
+ Toluene to make 4 L (Mallinckrodt #8608070)

7. Round bottom PVC 96-well microtiter plates (Dynatech #001-010-2401)

8. 12 X 100 mm polypropylene tubes + caps (Allied Fischer Scientific #14-956-7E and #14-956-15)

9. Test tube racks

10. p100 and p5000 pipetmen and tips

11. Centrifuge (Beckman #TJ-6 rotor TH4)

12. Scintillation vials (VWR #66022-300)

13. Scintillation counter (LKB Rackbeta #1211)

14. 37^{o}C incubator

15. Plastic box with cover, large enough to hold PVC plates. Place wet paper towels in the bottom = "moist chamber".

PROCEDURE:

1. Dilute AE-1 Ab to 10 ug/ml in HBS + azide (25 ul @ 4.7 mg/ml per 12 ml). Deliver 100 ug to each well of a microtiter plate. Cover with parafilm and incubate either overnight at 4°C or 3 hours at 37°C.

2. Discard plate contents and blot the plate on paper towels. Wash as follows: dip plate into a beaker containing 2L PBS-TX 100. Discard contents and blot the plate on a few paper towels. Repeat 2X, using separate beakers of PBS-TX 100 each time.

3. Fill all wells with 200 ul BSA/GEL/PBS and allow to sit at least 15 minutes.

4.

Stock AChE = 1 unit/ml
100 mU/ml = 20 ul @ 1 U/ml + 1.980 ml buffer

Activity (mU/ml)	ul @ 10mU/ml	ul buffer
1.5	150	850
1.0	100	900
0.75	75	925
0.50	50	950
0.25	25	975
0.10	10	990
0.00	0	1000

Test standard in triplicate and samples in duplicate.

5. Discard plate contents and deliver 100 ul sample or standard to appropriate well.

6. Cover with parafilm and incubate in moist chamber 3 hours at 37°C.

7. Wash as in 2.

8. Carefully transfer 100 ul "1x" 14C-AChE to each well. Cover with 2 sheets of parafilm and place in the moist chamber. Label "radioactive". Incubate overnight at 37°C (approximately 18 to 24 hours, noting actual time).

9. Label 12 x 100 tubes and carefully transfer 80 ul reaction volume to the appropriate tube.

10. Stop the reaction by injecting 2.0 ml stop buffer into each tube. Immediately add 5 ml scintillation cocktail, cap and shake vigorously.

11. Spin each tube at 1000 rpm for 10 minutes in the table top centrifuge (Equipment Room).

12. Using the p5000, carefully transfer 4.5 ml of the organic (upper) layer to a scintillation vial. Label CAP of vial.

13. Allow all vials to sit under the yellow cover of the scintillation counter at least 2 hours before counting. This reduces chem-iluminescence.

14. COUNT

To count:	Push	EDIT R
	Response	Parameter Group
	Push	04 R
	Response	ID
	Push	Date (Month/Day/Year)
	Response	Line #
	Push	1 R
	Response	Mode
	Push	2 R

You can ignore the remaining responses by pushing STOP R. Wait for the machine to respond with PASO, then push "START 04 R". Make sure there is an empty rack (no carrier vials, etc.) behind your samples. This tells the counter to STOP.